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F*rpo*: A Novel Single-Stranded DNA Promoter for Transcription and for Primer RNA Synthesis of DNA Replication

Hisao Masai* and Ken-ichi Arai
Department of Molecular and Developmental Biology
Institute of Medical Science
University of Tokyo
4-6-1 Shirokanedai
Minato-ku, Tokyo 108
Japan

Summary

We describe a novel promoter for E. coli RNA polymerase that functions efficiently only in the form of single-stranded DNA. Derived from the leading region of F plasmid, single-stranded F*rpo* sequence directs RNA polymerase to initiate transcription at a specific site within F*rpo*, and this specific transcription is highly stimulated by SSB. Prior denaturation activates transcription from otherwise inactive duplex DNA containing F*rpo*. Since RNAs synthesized on SSB-coated single-stranded F*rpo* are efficiently elongated into DNA chains by DNA polymerase III holoenzyme, transcription at F*rpo* serves also for priming DNA replication. A mode of recognition by RNA polymerase of a unique secondary structure within F*rpo* is proposed, and possible roles of this novel single-stranded promoter in expression and replication during conjugal transfer of F plasmid are discussed.

Introduction

Escherichia coli RNA polymerase recognizes promoter sequence elements centered about 10 and 35 residues proximal to transcription initiation sites on duplex DNA (O'Neil, 1989). It interacts with single-stranded DNA (ssDNA) in a rather nonspecific manner and initiates transcription randomly on ssDNA (Kaguni and Kornberg, 1982). The exceptions are complementary strand origins of single-stranded filamentous phages on which host RNA polymerase holoenzyme synthesizes a specific short RNA that is utilized as a primer RNA for DNA replication (Geider and Kornberg, 1974; Geider et al., 1978; Kaguni and Kornberg, 1982). This is the only RNA species synthesized from viral ssDNA, since these phages in the course of infection are converted to double-stranded DNA (dsDNA), which then serves as a transcription template for expression of viral proteins.

We have isolated a number of DNA sequences from duplex DNA genomes of various E. coli plasmid replicons that are capable of restoring clear plaque morphology when cloned as ssDNA on an M13 phage derivative that lacks the origin sequence for complementary strand synthesis (Nomura et al., 1982a, 1982b, 1991; Ray et al., 1982; Honda et al., 1989; Tanaka et al., 1991). These sequences, termed ssi for single-strand initiation sequences, can sustain efficient phage production presumably by providing the site for primer RNA synthesis and thereby facilitating replication. Enzymatic characterization of modes of primer RNA synthesis on these ssi revealed that they can be classified into two main groups (Masai and Arai, 1996): one that mediates multiple primings at scattered locations on the template DNA (Masai et al., 1990b, 1994; Masai and Arai, 1995) and another that provides priming at a specific site within an ssi (Masai and Arai, 1989; Masai et al., 1990a). We call the former type of priming mobile and the latter type immobile due to the nature of the protein complexes responsible for priming (Masai and Arai, 1996). In contrast to mobile priming, which requires action of multiple prepriming proteins before primer synthesis by primase, immobile priming requires a single protein that recognizes a priming signal and synthesizes a unique RNA primer. For example, G sites are recognized by primase and, in the presence of SSB, primer RNAs of defined length are synthesized (Masai and Arai, 1989; Masai et al., 1990b; Tanaka et al., 1991). Other examples of immobile priming are *ssiA* and *ssiB* of RSF1010 plasmid, on which the plasmid-encoded RepB′ protein synthesizes specific primer RNAs (Honda et al., 1989).

In the course of characterization of ssi, we identified a novel priming signal derived from F plasmid DNA that supported immobile priming of DNA replication. The primer RNA synthesis on this ssi is mediated by RNA polymerase (RNA polymerase) and, therefore, we designate it F*rpo*. In the presence of SSB, RNA polymerase efficiently initiates transcription at a specific site on ssDNA containing F*rpo*, and RNAs are elongated into DNA chains by DNA polymerase III holoenzyme. F*rpo* appears also to provide transcripts for downstream F plasmid open reading frames (ORFs), since the transcription initiation site on F*rpo* identified in vitro is identical to that previously localized in vivo for these ORFs. We present evidence that F*rpo* is a novel promoter for both mRNA synthesis and priming DNA replication that functions preferentially in the form of ssDNA. We also discuss possible functions of F*rpo* in expression and replication during conjugal transfer of F plasmid.

Results

SS (Circular ssDNA)-to-RF (Replicative Form DNA) Conversion from F Plasmid–Derived *ssiD*(F·f3) Is Dependent on Host RNA Polymerase

Previously, we reported 11 ssi isolated from various plasmid replicons (Nomura et al., 1991). Primer RNA could be synthesized on these ssi either by *dnaG*-encoded primase alone or by a primosome protein complex (Masai et al., 1990b, 1994; Masai and Arai, 1995). However, *ssiD*(F·f3), isolated from the EcoRI f3 fragment of F plasmid, could not support primer RNA synthesis by either of these enzymatic systems but was replicated efficiently in crude fraction II extract (Fuller et al., 1981) to produce fully replicated closed circular and nicked circular DNA (data not shown). This suggested that priming at *ssiD*(F·f3) utilizes a mechanism distinct from that for other ssi.

*To whom all correspondence should be addressed.

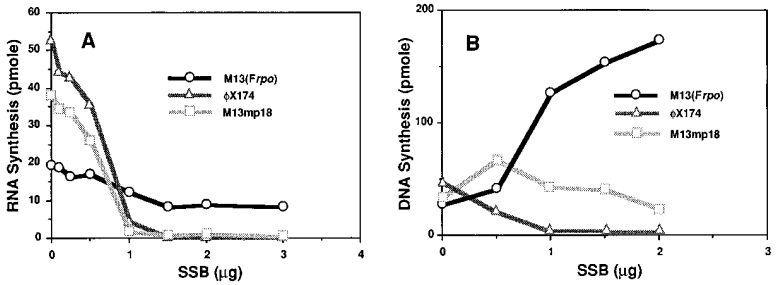

Figure 1. Effect of SSB on RNA and DNA Synthesis on Various SS Templates with Purified Proteins

Standard reaction mixtures (25 μl containing 0.1 μg template DNA) for in vitro RNA (A) and DNA (B) syntheses with RNA polymerase holoenzyme and DNA polymerase III holoenzyme were incubated in the presence of various amount of SSB. SS templates used are indicated.

The replication of SS containing *ssiD*(F·f3) in the crude extract was inhibited to background levels by addition of rifampicin at a concentration as low as 0.4 μg per ml (data not shown). Under the same reaction conditions, replication of øX174 phage DNA, known to be resistant to rifampicin, was not affected by this drug at a 1000-fold higher concentration. This result indicated that rifampicin-sensitive host RNA polymerase was required for priming *ssiD*(F·f3). We then measured DNA and RNA syntheses on *ssiD*(F·f3) by using purified proteins. Efficient DNA synthesis on SS containing *ssiD*(F·f3) could be reconstituted by SSB, RNA polymerase holoenzyme containing σ^{70} subunit (referred to hereafter as RNA polymerase holoenzyme) and DNA polymerase III holoenzyme (Table 1). On the basis of its requirement for RNA polymerase, we renamed *ssiD*(F·f3) F*rpo*, and the SS containing F*rpo* on the M13Δ*lac5* vector will be referred to as M13(F*rpo)*.

SSB Stimulates DNA Replication on M13(F*rpo*)

Reflecting nonspecific transcription by RNA polymerase on naked ssDNA, substantial amounts of RNA and DNA synthesis were observed on M13(F*rpo*) in the absence of SSB (Table 1). The effect of various concentrations of SSB on RNA synthesis by RNA polymerase holoenzyme was measured on three SS templates: M13mp18, øX174, and M13(F*rpo*). In the absence of SSB, significant RNA synthesis was observed on all three templates (Figure 1A). RNA synthesis on øX174 DNA was completely lost in the presence of a saturating level of SSB (1.5 μg SSB per 0.1 μg of template DNA; 0.7 μg of SSB should cover the entire ssDNA region of 0.1 μg template under the reaction condition [100 mM potassium glutamate], assuming that one tetramer of SSB covers 35 residues of ssDNA; Lohman and Ferrari, 1994), whereas a low but detectable RNA synthesis remained on M13mp18 in the same conditions (Kaguni and Kornberg, 1982). In contrast, addition of SSB only slightly reduced RNA synthesis on M13(F*rpo*), and a substantial level of RNA synthesis was detected in the presence of SSB at a level four times more than that required for coating all the SS in the reaction (3 μg of SSB per 0.1 μg of template).

Similarly, the low level of DNA synthesis observed on øX174 was completely suppressed by addition of SSB, while that on M13mp18 was stimulated nearly 2-fold at a subsaturating level of SSB and decreased to the level observed without SSB, as more SSB was added. In contrast, DNA synthesis on M13(F*rpo*) was stimulated nearly 6-fold by addition of a saturating level of SSB (Figure 1B). These results strongly suggest that RNA polymerase holoenzyme can synthesize specific transcripts on SSB-coated F*rpo* ssDNA and that these transcripts are efficiently elongated into DNA chains by DNA polymerase III holoenzyme.

Table 1. SS-to-RF Replication of M13(F*rpo*) with Purified Proteins

Protein Omitted	RNA Synthesis (pmol)	DNA Synthesis (pmol)
None	10	180
RNA polymerase	<0.1	2
DNA polymerase III holoenzyme	NA	<1
SSB	20	28

In vitro RNA or DNA synthesis was conducted with purified proteins on 300 pmol nucleotide of M13(F*rpo*) as described in Experimental Procedures except that the protein indicated was omitted. Amount of incorporation (pmol nucleotide) is shown. NA, not applicable.

RNA and DNA Products Synthesized on F*rpo*

The transcripts synthesized by RNA polymerase holoenzyme on M13(F*rpo*) were directly analyzed on a denaturing polyacrylamide gel. In the absence of SSB, a ladder of RNA transcripts ranging in size from several to several hundred residues was detected (Figure 2), reflecting nonspecific initiation and termination on the SS template. Addition of SSB suppressed most of the nonspecific transcription and only "specific" transcripts, whose synthesis were stimulated by the presence of a saturating level of SSB, remained. The prominent specific transcripts ranged in size from 85 to 155 residues, with the most intensive bands being 116 to 122 residues. Some of these transcripts (151 to 155) were barely detectable in the absence of SSB. The relatively intense bands (two doublets) near the top of the right panel of Figure 2 are 330–360 residues and even larger transcripts were readily detected. In sharp contrast, only a single 20-residue transcript was synthesized on SSB-coated M13mp18 (data not shown; Van Wezenbeek et al., 1980; Higashitani et al., 1996). When transcription was conducted on M13(F*rpo*)-SSB complexes that had been isolated by gel filtration, a similar profile of specific transcripts was observed (data not shown). Our results indicate that F*rpo* coated by SSB can support RNA synthesis with purified RNA polymerase and that the profile of the RNA transcripts significantly differs from those synthesized on SSB-coated filamentous phage DNAs.

DNA products synthesized on M13(F*rpo*) by purified proteins were full-length nicked circular DNA (7.4 kb), which appeared at 2 min after incubation was initiated

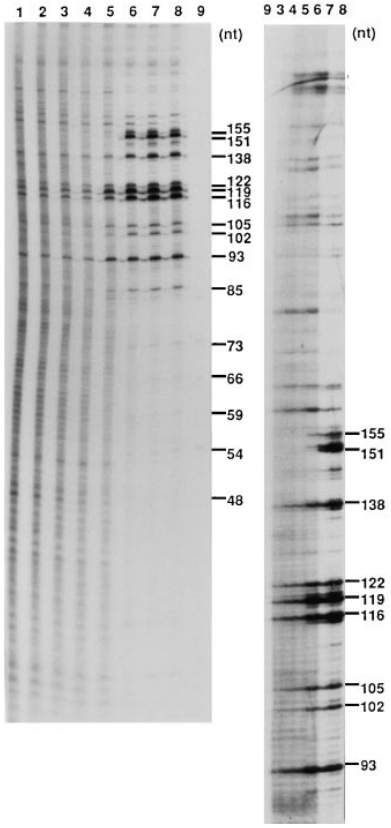

Figure 2. RNA Products Synthesized on F*rpo*

RNA was synthesized in standard reaction mixtures for in vitro RNA synthesis in the presence of various amounts of SSB. Amount of SSB present in each reaction mixture was (for both left and right panels): 0 μg (lanes 1 and 9), 0.1 μg (lane 2), 0.25 μg (lane 3), 0.5 μg (lane 4), 1 μg (lane 5), 1.5 μg (lane 6), 2 μg (lane 7), and 3 μg (lane 8). RNA polymerase was not added in lane 9. The right panel shows a part of the same samples run for a longer period to obtain better resolution of higher molecular weight products.

and whose level increased up to 10 min (data not shown). RNA products synthesized in a reaction coupled to DNA synthesis were almost identical to those synthesized in the absence of DNA synthesis, suggesting that they are utilized as primers for DNA replication (data not shown). We conclude that F*rpo* supports RNA polymerase-dependent priming of DNA replication in the presence of SSB.

RNA Synthesis on M13(F*rpo*) Requires RNA Polymerase Holoenzyme

Isolated SSB-M13(F*rpo*) complexes were assayed for RNA and DNA synthesis either with RNA polymerase holo- or core enzyme. Efficient RNA and DNA synthesis were both dependent on holo RNA polymerase containing σ^{70} subunit (data not shown); core RNA polymerase only supported a low level of synthesis. Since core enzyme is known to have higher affinity to ssDNA, this low level of synthesis could be due to nonspecific initiation of transcription from the template region, which still remained uncoated by SSB.

Primer RNA Synthesis Initiates at a Specific Site on F*rpo*

In order to locate the site of priming, DNA products synthesized in vitro in the crude extract on M13(F*rpo*) were digested with a restriction enzyme and run on an alkaline agarose gel. If priming for replication is at a specific position on the template DNA, additional fragments derived from the restriction fragment containing the nick corresponding to the initiation site should appear on an alkaline-denaturing gel (Figures 3A and 3B) (Horiuchi and Zinder, 1976). The products containing nicked or gapped circular DNA were digested either by ClaI or XmnI. There are two sites for each of these enzymes on the template DNA; ClaI should generate 3.0 kb and 4.4 kb fragments, while XmnI should yield 2.3 kb and 5.1 kb fragments. If the nick is present within the F*rpo* region (354 bp), two ClaI fragments of 3.4–3.8 kb and 0.6–1.0 kb length or XmnI fragments of 3.3–3.7 kb and 1.4–1.8 kb length should be generated on an alkaline gel (Figure 3A). In keeping with this expectation, two additional fragments of the predicted sizes appeared in each enzyme digestion (Figure 3B). The low intensities of the smaller DNA fragments, which are derived from the region 3′ to the putative initiation site, may indicate that DNA chain elongation may have terminated before it came to completion in some fractions of the products. The result suggests that the initiation of DNA replication occurs at a defined site in or near F*rpo*.

In order to more precisely localize the site(s) of priming, the restriction enzyme–digested products were analyzed on a denaturing polyacrylamide gel. Products were digested either with NgoMI or with BanII, each of which should cleave the template DNA at a single site. These two restriction sites are 26 nucleotides apart (Figure 3A). Fragments from the 5′ ends of nascent DNA to each respective restriction site were identified on the gel. The patterns of fragments generated by the two enzymes were nearly identical except that the fragments generated by NgoMI were longer by 26 nucleotides, in agreement with the distance of the two sites (Figure 3C) and indicating that these fragments are actually derived from the 5′ ends of the nascent DNA. The major 5′ end of the nascent DNA fragment was localized at position 6 based on the size of the most intense DNA fragment in comparison with a marker DNA (Figure 3C). RNase A treatment of the products prior to gel electrophoresis did not change the sizes of the fragments, indicating that RNA species were not attached to the 5′ termini of the DNA fragments (data not shown). This is probably because the primer RNA is removed by 5′-to-3′ exonuclease activity of DNA polymerase I or by RNase H in the crude extract. Therefore, position 6 appears to be the RNA–DNA transition site, rather than where the priming is initiated.

In order to localize the 5′ end of primer RNA synthesized by purified RNA polymerase, the products synthesized by purified RNA polymerase and DNA polymerase III holoenzyme were analyzed in a similar manner. In the purified system, primer RNAs are expected to be left intact at the 5′ end of the nascent DNA chains. NgoMI or EcoRI digestion generated ~520 and 113 bp fragments, respectively (Figure 3D, lanes 1 and 3). This localized the 5′ end of the primer RNA at the G residue of position

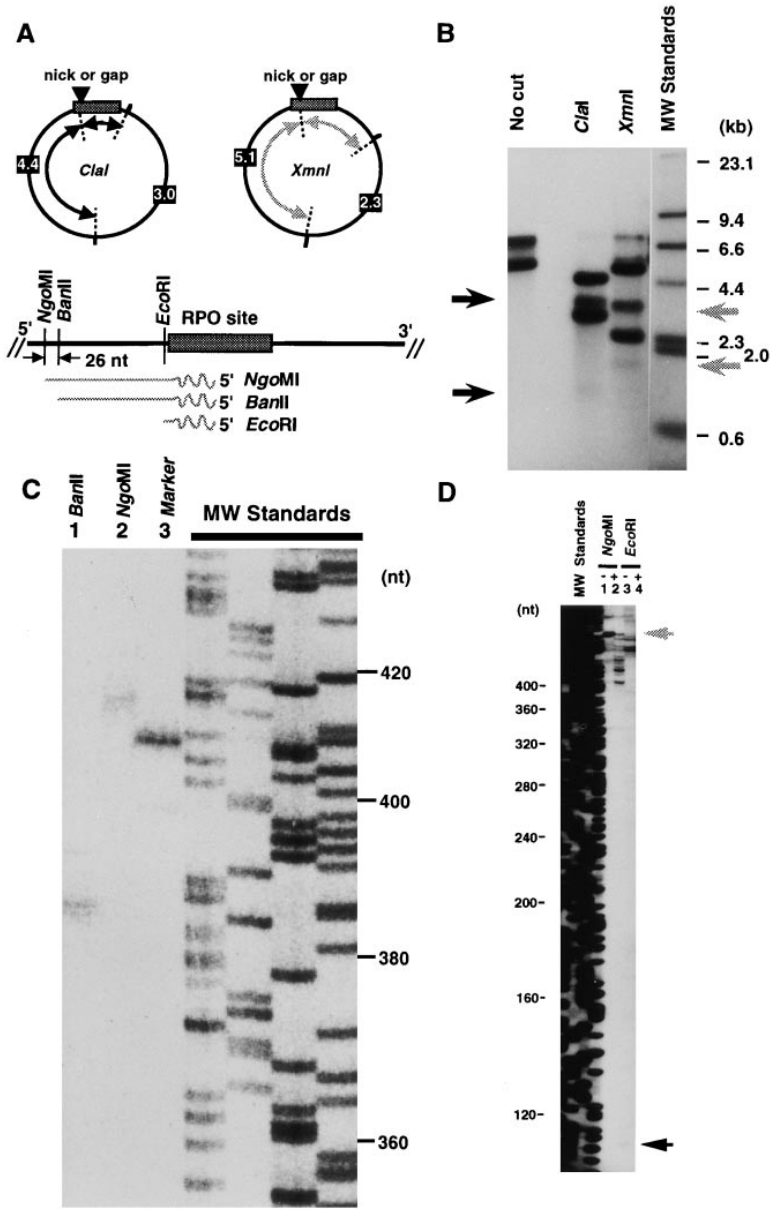

Figure 3. Mapping of Initiation Site of DNA Replication on F*rpo*

(A) Locations of relevant restriction sites on M13(F*rpo*). (Upper) The shaded bars indicate F*rpo* sequence cloned on the vector with a potential nick site (generated as a result of initiation) indicated as an arrowhead. The numbers indicate the expected length (in kb) of the restriction fragments generated by digestion. The black (for ClaI) and gray (for XmnI) double-arrowed arcs represent the expected ssDNA fragments generated by digestion due to the presence of the nick in the insert, which are indicated in (B) by the arrows of the same colors. (Lower) Locations of BanII, NgoMI, and EcoRI sites are indicated on the bar representing a part of the vector sequence. The nascent DNA chain and attached primer RNA are indicated by gray line and gray wavy line, respectively. The fragments generated by digestion with BanII or NgoMI are expected to differ in size by 26 nt. (B) The products containing both closed circular and nicked or gapped circular DNA synthesized in fraction II on M13(F*rpo*) were digested by ClaI or XmnI and were analyzed on a 0.8% alkaline agarose gel as described in Experimental Procedures. Molecular weight markers are λ DNA digested with HindIII. Arrows (black for ClaI and gray for XmnI) indicate the DNA bands expected from a unique initiation of DNA replication within F*rpo*. kb, kilobase. (C) The products synthesized in fraction II on M13(F*rpo*) were digested either by BanII (lane 1) or NgoMI (lane 2) and were analyzed on a 5% polyacrylamide gel containing 8 M urea as described in Experimental Procedures. The nascent DNA fragments encompassing from the initiation site of DNA chain elongation to the respective restriction sites appear on the gel. Lane 3: size control DNA fragment from the NgoMI site (labeled at this site by fill-in) to the EcoRI site, located at the 5′-proximal end of the F*rpo* insert. NgoMI or BanII digestion of the product generated 413/412 or 387/386 nt long DNA fragment, respectively. (D) The products synthesized with purified proteins were digested either by NgoMI (lanes 1 and 2) or by EcoRI (lanes 3 and 4) and were analyzed on a 6% polyacrylamide gel containing 8 M urea either before (−) or after (+) RNase A digestion. Gray (lane 1) or black (lane 3) arrow indicates the RNA primer-associated nascent DNA fragment encompassing through the NgoMI or EcoRI site, respectively. MW standards in (C) and (D) were the dideoxy sequencing ladder on M13mp18 with 5′-phosphorylated "−40" universal primer and that on M13(F*rpo*) with "−20" primer, respectively. nt, nucleotide.

111. Prior RNase A digestion generated a ladder of DNA fragments over a range of ~160 bp, indicating the presence of RNA primers of varied lengths (Figure 3D, lane 2). Some of the transition sites are located within the vector sequence, indicating that the site selection for the RNA–DNA transition in the purified system is not specific to F*rpo* sequences. The length distribution of RNA primers attached to the nascent DNA chains estimated from RNase A digestion is similar to that of RNA transcripts synthesized in an uncoupled reaction (i.e. by RNA polymerase alone). Furthermore, the profile of the RNA transcripts synthesized in a reaction coupled with DNA synthesis was nearly identical to that observed in an uncoupled reaction (data not shown). Finally, primer extension on RNA synthesized on F*rpo* localized the 5′ end at AAG (position 111 to 109 on the + strand), consistent with the restriction digestion mapping described above (Figure 4A, lane 2). Transcription was initiated at this site even in the absence of SSB, although addition of SSB enhanced the level of transcription by more than 5-fold (Figure 4A, compare lanes 2 and 4). These results demonstrate that transcription by RNA polymerase on SSB-coated M13(F*rpo*) is initiated at a specific site and is terminated at multiple locations, thus

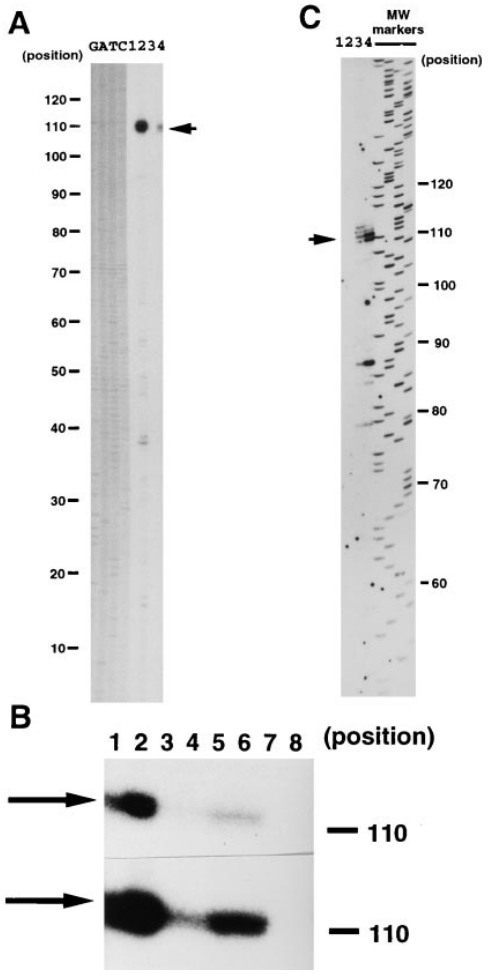

Figure 4. Transcription on Duplex DNA Containing F*rpo*: Primer Extension and Run-Off Transcription

(A) Primer extension was conducted on RNA synthesized on M13(F*rpo*) in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of SSB (1 μg per 0.1 μg of template) as described in Experimental Procedures. Lanes 1 and 3, control primer extension reaction on prior transcription reaction mixtures without RNA polymerase. Products were analyzed on an 8% polyacrylamide gel containing 8 M urea. (B) Primer extension of RNA synthesized in vitro on M13(F*rpo*) (lanes 1 and 2), M13(F*rpo*) RF (lanes 3 and 4), heat-denatured M13(F*rpo*) RF (lanes 5 and 6), and HindIII (present at the 3′-proximal end of the F*rpo* insert)-digested M13(F*rpo*) RF (lanes 7 and 8). RNA was synthesized in the presence (lanes 2, 4, 6, and 8) or the absence (lanes 1, 3, 5, and 7) of SSB. The lower part is a longer exposure of the same gel to show the weak bands in lanes 3 and 4. (C) Run-off transcripts synthesized on isolated 365 bp ds EcoRI-HindIII fragment derived from RF DNA containing F*rpo*. Prior to RNA synthesis, template DNA was heated in lanes 3 and 4. RNA was synthesized in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of SSB (1 μg per 0.1 μg of template). RNAs were analyzed on an 8% polyacrylamide gel containing 8 M urea. Molecular weight markers were the dideoxynucleotide sequencing ladder on RF DNA of M13(F*rpo*) with 5′-phosphorylated reverse primer (A and B) and that on M13mp18 with 5′-phosphorylated "−40" universal primer (C).

generating transcript size heterogeneity. The 3′ hydroxyl groups of these transcripts can be utilized by DNA polymerase III holoenzyme for DNA chain elongation. The predominant transition from RNA to DNA at position 6 in the crude extract suggests the existence of a "specificity factor" that determines the position of RNA–DNA transition within F*rpo*.

F*rpo* Is a Single-Stranded DNA Promoter

Synthesis of long RNA species on M13(F*rpo*) in the presence of SSB (Figure 2) suggests that these RNAs could serve as mRNA for translation. In fact, two ORFs, ORF95 and ORF273, were previously identified in the leading region of F plasmid, and they are present immediately downstream of F*rpo* (Loh et al., 1989). The transcription initiation site for these ORFs maps in vivo to the A residue of position 110 (Loh et al., 1989), nearly identical to where we determined that RNA polymerase initiated transcription on M13(F*rpo*) in vitro. Therefore, we considered a possibility that F*rpo* might function in transcribing these ORFs.

We analyzed transcription on duplex DNAs containing F*rpo* by primer extension. On supercoiled RF DNA containing F*rpo*, transcription was initiated at the site identical to that observed on ssDNA, although the efficiency was significantly lower than transcription on ssF*rpo* (Figure 4B, lane 3). The presence of SSB, which stimulated F*rpo*-dependent transcription on SS (Figure 4A, lanes 2 and 4; Figure 4B, lanes 1 and 2), did not stimulate transcription on RF (Figure 4B, compare lanes 3 and 4). Prior denaturation of dsDNA template resulted in a 3-fold increase in specific transcription (Figure 4B, lane 5), which was not further stimulated by addition of SSB (Figure 4B, lane 6). Linearized RF DNA was totally inactive in the presence and absence of SSB (Figure 4B, lanes 7 and 8). In keeping with this result, transcription was not detected on the isolated dsDNA fragment containing the F*rpo* sequence in run-off transcription assays (Figure 4C, lanes 1 and 2). However, it was activated by prior heat denaturing of the template DNA (Figure 4C, lane 3) and was further enhanced by addition of SSB (Figure 4C, lane 4). The major transcription initiation sites on the denatured DNA fragment were mapped to positions 110–113, nearly identical with transcription on SS. These results indicate that F*rpo* is a promoter that functions in the form of single-stranded DNA. The weak activity observed on supercoiled DNA probably reflects partial melting of the duplex DNA due to negative superhelicity, which would permit recognition of the promoter by RNA polymerase holoenzyme.

Secondary Structure of F*rpo* and Mode of Interaction with RNA Polymerase

Binding of SSB to unstructured ssDNA generally inhibits binding of other ssDNA binding proteins. The stimulatory effect of SSB on RNA polymerase-mediated transcription on M13(F*rpo*) suggests the presence of a secondary structure that may be important for recognition by RNA polymerase. On the basis of the results of nuclease digestion and protection experiments, we present a possible long stem and a loop secondary structure of F*rpo* (Figures 5 and 6, data not shown).

Extensive cleavages by DNase I, which attacks dsDNA more preferentially over ssDNA, support the presence of a secondary structure within F*rpo* (see Figure 5A).

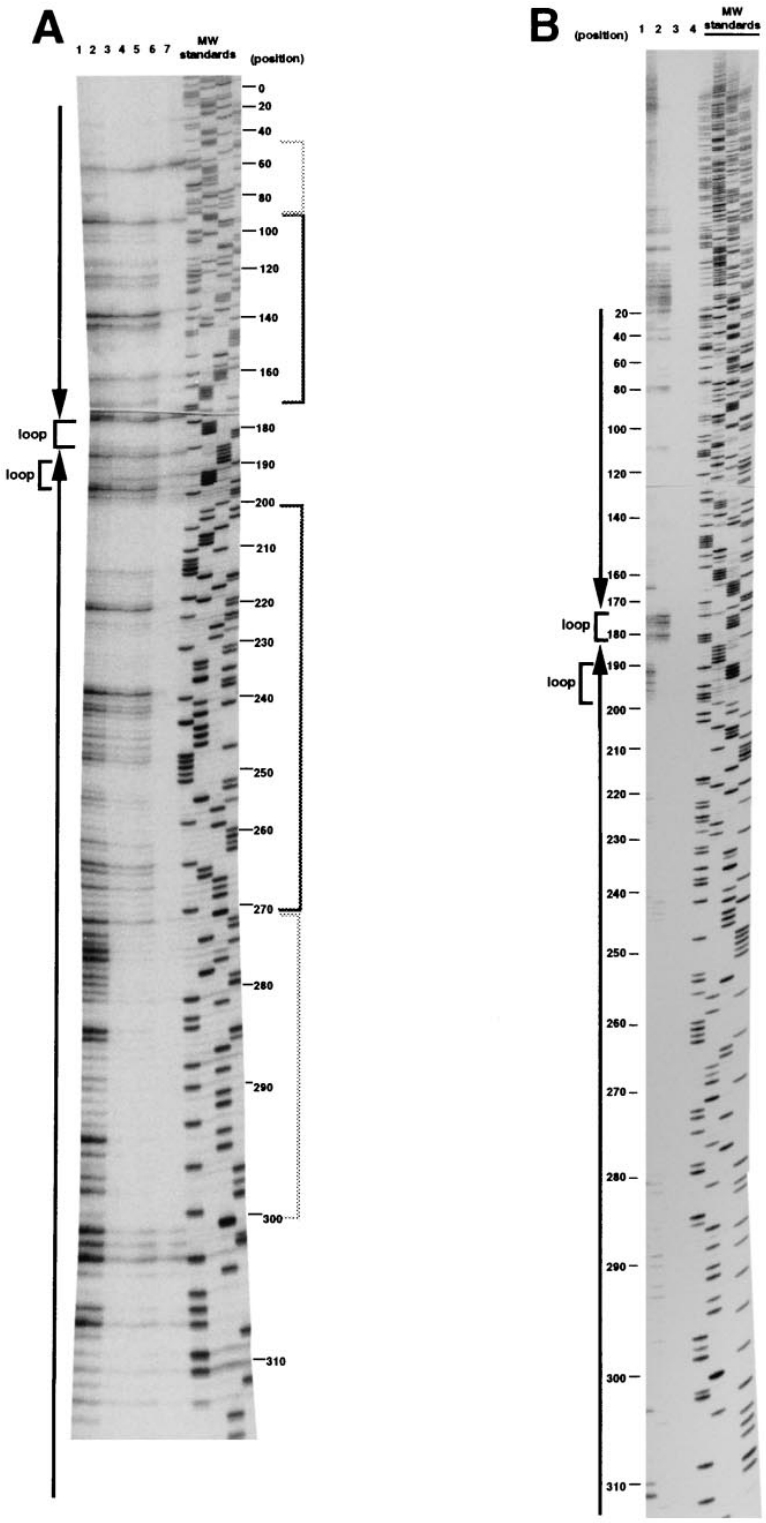

Figure 5. DNase I Footprinting Analysis and P1 Nuclease Mapping of F*rpo*

(A) DNase I protection assay was performed on M13(F*rpo*) as described in Experimental Procedures. The amount of SSB in the reaction mixtures were: 0 μg (lane 1), 0.5 μg (lane 2), and 1 μg (lanes 3–7). RNA polymerase holoenzyme added was 0 ng (lanes 1–3), 68 ng (lane 4), 135 ng (lane 5), 270 ng (lane 6), and 675 ng (lane 7). The regions protected by RNA polymerase or by SSB are shown by two dark gray or light gray brackets, respectively. (B) P1 nuclease mapping was conducted as described in Experimental Procedures. DNA was digested by the following amount of P1 nuclease: lane 1, 0.01 unit; lane 2, 0.1 unit; lane 3, 1 unit; lane 4, no nuclease. For both (A) and (B), MW standards were the dideoxynucleotide sequencing ladder on M13(F*rpo*) with 5′-phosphorylated "−40" primer, and the samples were run on a 6% polyacrylamide gel containing 8 M urea. Two converging arrowed lines and the small brackets indicate the extent of the base-paired stem sequences and the locations of the proposed loops, respectively.

Clusters of sites sensitive to DNase I are present on the stem, and they are located at defined loci on both stem strands, whereas the loop region (position 173–181) was not digested by DNase I even at a high nuclease concentration (Figure 5A). In contrast, more localized cleavages were detected by single-strand-specific nuclease P1. At an optimal nuclease concentration, two large loops at the top of the hairpin (positions 173–181 and 186–199) were most sensitive to P1 nuclease (Figure 5B, lanes 1 and 2). Other weak P1-sensitive sites consistently located to the unpaired nucleotides. These results support the proposed secondary structure of F*rpo*. Addition of

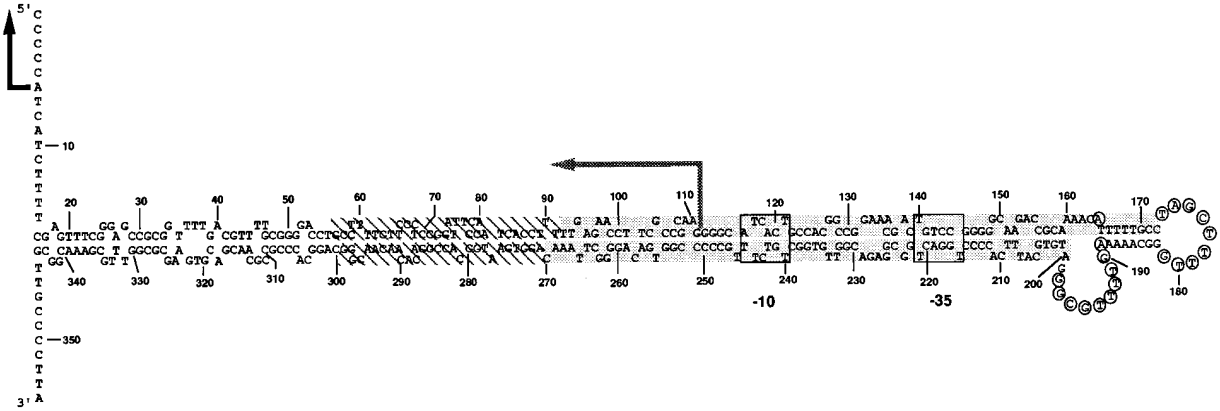

Figure 6. Proposed Secondary Structure of ssF*rpo* and a Possible Mode of Recognition by RNA Polymerase

Nucleotides protected from DNase I digestion are indicated by gray (for RNA polymerase) and by striped (SSB) boxes. Gray and black arrows indicate, respectively, the position of the major 5′ end of the primer RNA synthesized in the purified system and that of the transition site from RNA to DNA in the crude extract. The arrows indicate the direction of transcription/DNA chain elongation. The residues sensitive to P1 nuclease near the top of the stem are circled. Putative −10 and −35 sequences are boxed.

SSB did not significantly affect the pattern of DNase I sensitivities except for partial protection of sequences 55–90 and 270–299 (Figure 5A, lanes 2 and 3, and Figure 6), indicating that F*rpo* spontaneously adopts a secondary structure that is not melted by binding of SSB.

Addition of RNA polymerase holoenzyme to SSB-coated M13(F*rpo*) generated two distinct areas of strongly protected regions, which spanned from position 92 to 170 and from 198 to 269. Assuming the transcription initiation site as +1, they corresponded to −60 to +20 on the upper (template) strand and −51 to +20 of the lower strand (Figure 6). The sequences between the two protected regions remained sensitive to DNase I even at the highest concentration of RNA polymerase. The observed protection is consistent with the proposal that RNA polymerase binds to the stem and initiates transcription in the middle of the stem toward its bottom with the upper strand being utilized as a template. When the core enzyme was used instead of holoenzyme, the protection was much weaker even at high enzyme concentrations (data not shown), consistent with inefficient transcription by the core enzyme.

Discussion

Isolation of F*rpo* as a Signal for Primer RNA Synthesis

F*rpo* [previously called *ssiD*(F·f3)] was isolated from the genome of F plasmid on the basis of its ability to complement poor growth of an origin-defective M13 phage vector (Nomura et al., 1991). F*rpo* is recognized by host RNA polymerase and supports primer RNA synthesis at a specific site. Therefore, it belongs to the ''immobile'' category of priming signals. RNA species synthesized on M13(F*rpo*), which are heterogeneous in size, were elongated into DNA chains by DNA polymerase III holoenzyme. Primer RNA synthesis initiated at a specific site (position 111) within F*rpo*. The profile of the RNA species synthesized on SSB-coated M13(F*rpo*) by RNA polymerase alone is nearly identical with those synthesized in the presence of DNA synthesis. Furthermore, the locations of multiple RNA–DNA transition sites are consistent with those of the expected 3′ ends of the heterogeneous RNA transcripts initiated at the specific site. These results suggest that RNA polymerase specifically recognizes SSB-coated F*rpo* and initiates transcription exclusively at the position 111. This specific transcription is terminated at multiple positions and this generates the RNA transcript size heterogeneity. Much of the transcription termination, and therefore RNA–DNA transition, occurs within flanking vector sequence, indicating that site selection of RNA–DNA transition is not determined by specific sequences on F*rpo* in reactions driven by purified RNA polymerase and DNA polymerase III holoenzyme. DNA chains appeared to be casually elongated from any 3′ hydroxyl termini of the RNA transcripts generated. The intrinsic ability of F*rpo* to retain the RNA transcripts on the DNA template as RNA–DNA hybrids may contribute to efficient chain elongation by DNA polymerase III holoenzyme.

In contrast, a major 5′ end of DNA chains was mapped within F*rpo* in the crude fraction II extract. RNase A treatment of the products did not change the lengths of the fragments, showing the absence of RNA primers at the 5′ end of the nascent DNA fragments. They probably have been removed by cellular RNase H or by the 5′-to-3′ exonuclease activity of DNA polymerase I present in fraction II. Although the position of the 5′ end of the primer RNA synthesized on M13(F*rpo*) in fraction II could not be determined, it is likely that RNA polymerase initiates transcription in the crude extract at the site identical to that mapped in the purified system. The predominant 5′ end of nascent DNA chains at position 6 in the crude extract may indicate the existence of an additional cellular factor(s) that directs the RNA–DNA transition at this position. This factor may terminate the transcription at a specific site on F*rpo*. Alternatively, the factor may process the elongated RNA transcripts at a defined site, as RNase H does to generate a mature RNA primer for leading strand synthesis of ColE1-type plasmids (Itoh and Tomizawa, 1978, 1980). Addition of

purified RNase H to the in vitro replication assays, however, did not result in RNA–DNA transition at the specific site on F*rpo*. Addition of a small amount of the crude extract made the transition specific (data not shown). Further purification of this factor(s) will clarify its nature.

Stimulation of Specific Transcription on ssF*rpo* by SSB

Transcription of the specific transcripts as well as DNA replication on M13(F*rpo*) was strongly stimulated by SSB. How does SSB stimulate specific transcription on these sequences? The patterns of cleavage of F*rpo* DNA by DNase I or by P1 nuclease were not significantly affected by SSB, suggesting that generation of a secondary structure is intrinsic to F*rpo* and that it is not melted by SSB (Figures 5 and 6; data not shown). Therefore, SSB may facilitate the recognition by RNA polymerase by stabilizing the secondary structure, although a subtle change of the secondary structure of F*rpo* induced by binding of SSB could play a critical role in this RNA polymerase recognition. An alternative possibility is that interaction of SSB with RNA polymerase facilitates recognition of F*rpo* by the latter protein. In DNase I protection experiments, we observed partial but reproducible protection of −21 to −59 sequences (relative to the +1 initiation site) of the proposed stem by SSB binding (Figures 5 and 6). SSB may induce localized melting of the stem in this region and bind to the melted ssDNA. The putative SSB binding site lies adjacent to the RNA polymerase binding site (−20 to +60, Figure 6), raising an intriguing possibility that SSB may facilitate binding of RNA polymerase to F*rpo* through protein–protein interaction on the template DNA. Recent studies indicate that interaction of RF-A, the eukaryotic counterpart of SSB, with transcription factors including E2 and VP16, is likely to explain the stimulation of replication by the transcription enhancers present near the origin sequences of viral replicons (Dutta, 1993). RF-A also interacts with the viral initiator proteins, T-antigen and E1 (Melendy and Stillman, 1993). Therefore, although it has been assumed that SSB is required for DNA replication simply to prevent reannealing of unwound DNA templates, a more active role of SSB in initiation of DNA replication at origins could be envisioned as a conserved feature for both prokaryotic and eukaryotic DNA replication. SSB is also essential for primer RNA synthesis by primase at the complementary origin of G4 phage and plasmid-derived G sites (Bouche et al., 1978; Masai et al. 1990a). Possible interaction of SSB with primase could also explain the crucial function of SSB in these primase-dependent primer RNA syntheses.

Mode of Recognition of F*rpo* by RNA Polymerase

Patterns of P1 cleavages and DNase I protection are consistent with the proposed large stem-loop structure, whose stem may be recognized and bound by RNA polymerase (Figures 5 and 6). Efficient priming and DNA replication requires RNA polymerase holoenzyme containing σ^{70} subunit. Consistent with this, the holoenzyme, but not the core enzyme, bound specifically to SSB-coated M13(F*rpo*) (data not shown). Therefore, the σ factor may play a critical role in recognition of F*rpo*. In the stem, −10 and −35-like sequences were identified (5′-TGTCTT-3′/5′-ATCACT-3′ and 5′-TGGACT-3′/5′-TGTCC*-3′; the residues matching with the consensus are underlined, see Figure 6). We were surprised that quite a few mismatches in the stem can be tolerated for RNA polymerase recognition of these structures. Similar flexibility in baseparing appears to be the case for filamentous phage origins where mutational analyses of the −10 sequence of the phage origin indicated the requirement for specific sequences rather than basepairing (Higashitani et al., 1996). Similarly, the putative −10 sequence for F*rpo* contains only three base-paired nucleotides out of six nucleotides. Recently, it was reported that the rate of open complex formation can be generally increased by the presence of unpaired bases in the −10 region, as long as specific base sequences at positions −7 and −11 are maintained (Roberts and Roberts, 1996). Relative dispensability of basepairing in the spacer region between the −10 and −35 sequences (7 out of 18) is also consistent with the filamentous phage promoter, in which 12 out of 18 nucleotide spacers are a single strand linking the two hairpins (Higashitani et al., 1993, 1996). More detailed study on protein–DNA interaction as well as mutation analyses would not only clarify the precise mode by which RNA polymerase recognizes this unique ssDNA promoter, but also provide novel insights into how RNA polymerase initiates transcription at conventional σ^{70} promoters.

The length and distribution of RNAs synthesized by RNA polymerase on F*rpo* are quite different from those on the complementary strand origin of filamentous phages (Geider et al., 1978; Higashitani et al., 1993, 1996). A 20-residue RNA transcript is the major product synthesized at the origins of single-stranded filamentous phages. In contrast, on SSB-coated M13(F*rpo*) the majority of the transcripts are longer than 85 residues and shorter transcripts are rare. This probably reflects the structure of each DNA. Recent remodeling of the complementary strand origin of a filamentous phage indicates the presence of a double hairpin that provides −35 and −10 sequences for recognition by RNA polymerase (Higashitani et al., 1996). Transcription on this double-hairpin ends after a 20-residue transcript is synthesized when RNA polymerase reaches the top of the hairpin. Transition from RNA to DNA occurs at this site in the absence of any additional factor. On the other hand, transcription on F*rpo* proceeds toward the bottom of the hairpin stem and can continue on the template strand, generating the observed longer transcripts of varied lengths.

F*rpo* Is an ssDNA Promoter for mRNA Transcription as well as for Priming DNA Replication

Promoter activity was previously detected on the F plasmid SmaI–EcoRI fragment (coordinate 64.2F–64.7F) that contains F*rpo* (Cram et al., 1984). Furthermore, in cells harboring a duplex plasmid containing the leading region of F plasmid, the transcription initiation site for ORF95 and ORF273, present downstream of F*rpo*, maps to position 110, nearly identical to that mapped in vitro for transcription from F*rpo* (Cram et al., 1984; Loh, et al., 1989). Extensive elongation of transcripts on SSB-coated single-stranded M13(F*rpo*) is consistent with the

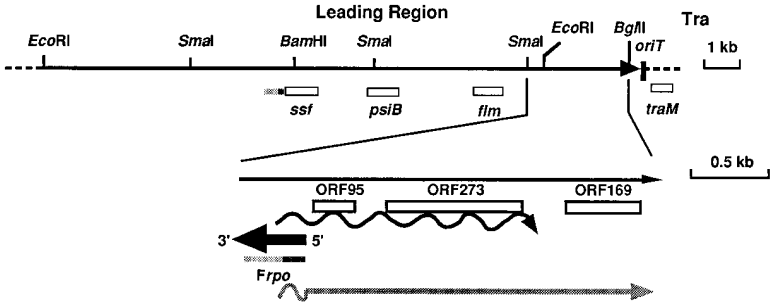

Figure 7. Locations of F*rpo* and a F*rpo*-like Sequence on the F Plasmid Genome

F*rpo*, the template strand of which is shown as a thick black arrow in 5′-to-3′ direction, is present ~2.1 kb away from *oriT*, the nick site of transfer DNA replication. During conjugation, one strand of the F plasmid containing the leading region is transferred from the nick in the direction shown as the arrows at the ends of the thin black lines (from 5′ to 3′). F*rpo*, present on the transferred strand, may direct transcription toward ORF95 and ORF273, as shown by the black arrowed wavy line. Alternatively, DNA between F*rpo* and the nick site can be converted into duplex, as shown by the gray arrowed line continuing from the gray wavy line, if transcription is coupled to DNA synthesis. The sequences identical (137 nt long) or similar to that of F*rpo* are present upstream of the *ssf* coding region, and its extent is indicated by the dark gray and light gray bars representing the regions which are 100% and 50% identical, respectively. Open boxes indicate open reading frames, under which genes encoded are shown, where known. Tra contains the transfer operon.

notion that these transcripts can serve as mRNA for expression of proteins. This strongly suggests that F*rpo* is a functional promoter for expression of ORF95 and ORF273. However, F*rpo* is not an active promoter in vitro when it is present on duplex DNA, although it can be activated by prior heat denaturing of duplex DNA (Figure 4). These results indicate that F*rpo* not only promotes replication by supplying primer RNAs but also is a unique promoter for protein expression, which functions efficiently only when single-stranded.

F*rpo* is an example of an E. coli promoter that primarily functions as ssDNA. An N4 phage–derived sequence, which is recognized by the phage-encoded RNA polymerase, was previously reported to be functional only when it was present on a supercoiled DNA or as ssDNA (Glucksmann et al., 1992; Markiewicz et al., 1992). In this case, SSB was stimulatory for transcription on supercoiled DNA containing the phage promoter. In our case, SSB stimulated transcription of F*rpo* only on ssDNA. We do not know the reason for this difference.

F*rpo* May Promote Gene Expression and DNA Replication during Plasmid Conjugation

What are possible functions of F*rpo* in replication and gene expression of F plasmid? F*rpo* is present near *oriT*, the initiation site of F transfer DNA replication (Thompson et al., 1984; Willetts and Wilkins, 1984). F transfer DNA replication is initiated by a nick at *oriT* and proceeds by a rolling circle–type mode (Frost et al., 1994). ssDNA nicked at *oriT*enters recipient cells from the 5′ end in the direction shown in Figure 7 (Loh et al., 1989) and the transferred ssDNA is converted into duplex DNA in the recipient cell. F*rpo* is present on the transferred strand, and it is one of the earliest regions of F DNA that is transferred into the recipient. F*rpo* can prime DNA replication and convert the DNA region at the end of the transferred strand into dsDNA in the recipient cells. F*rpo* can also provide an mRNA for the two ORFs, ORF95 and ORF273, which are conserved in the *tra* regions among different conjugative plasmids and may encode proteins that play important roles in conjugation (Loh et al., 1989). These two genes may be efficiently transcribed within the recipient cells before the transferred strand is converted to double strand, although this has not yet been experimentally shown. It was reported that expression of ColIb-encoded *ssb* and *psiB* genes as well as that of F-encoded *psiB* is sharply induced in the recipient cells shortly after conjugation (Jones et al., 1992; Bagdasarian et al., 1992). These two genes appear to be cotranscribed from the *ssb* promoter, and their locations and structures are highly conserved in various conjugative plasmids (Frost et al., 1994). We have discovered that the promoter region of *ssf* (encoding the SSB of the F plasmid; Chase et al., 1983) shares 100% identity with the 5′-proximal 137 nucleotides of F*rpo*. The zygotic induction of *ssb* (and possibly *ssf*) and *psiB* could be readily explained by activation of this F*rpo*-like promoter during transfer of ssDNA into recipient cells.

In conclusion, we have identified and characterized a novel E. coli promoter, which functions in the form of a single strand specifically activated by SSB, and we suggest a possibility that it functions in zygotic induction of gene expression and DNA replication during conjugal transfer.

Experimental Procedures

Strains, DNA, and Reagents

For plaque morphology assays, E. coli K12 strains K37 (Hfr, *supD*) and JM103 (*supE*, *thi*, *hsdR4*Δ[*lac-proAB*], F′[*traD36*, *proAB*$^+$, *lacI*q, lacZΔM15]) were used as hosts. For plasmid construction, DH5α (MC1061 [*hsdR*, *mcrB*, *araD139*, Δ(*araABC-leu*]7679, Δ*lacX74*, *galK*, *rpsL*, *thi*) and WM1100 (MC1061, *recA::Tn10*) were used as host strains. Fraction II extracts were prepared from C600 as previously described (Fuller et al., 1981). M13(F*rpo*), previously called F-3205, contains a 354-nucleotide insert derived from the EcoRI f3 fragment of F plasmid into the vector M13Δ*lac*5. F*rpo* was previously called *ssiD*(F·f3) (Nomura et al., 1991).

RNA polymerase holo- and core enzymes were gifts from Dr. Akira Ishihama. SSB was purchased from United States Biochemical, Inc. DNA polymerase III holoenzyme was a gift from A. Kornberg's laboratory.

In Vitro DNA Replication in the Fraction II Extract

Reaction mixtures (25 μl) for SS-to-RF replication in fraction II contained 40 mM HEPES–KOH (pH 7.6), 100 mM potassium glutamate, 8 mM magnesium acetate, 4 mM dithiothreitol, 80 μg/ml bovine serum albumin, 2 mM ATP, 50 μM each of CTP, GTP, and UTP, 1% (w/v) polyethylene glycol 8000, 100 μM creatine phosphate, 100 μg/ml creatine kinase, 100 μM each of four deoxynucleotides with [α-^{32}P]TTP (200–500 cpm/pmol of deoxynucleotides), 100 ng or 0.15 μg (equivalent to 300 or 450 pmol, respectively, of nucleotides) of SS template, and 100–150 μg protein in fraction II. Incubation was 10 min at 30°C. DNA synthesis was quantitated by measuring acid-insoluble radioactivity as previously described (Fuller et al., 1981).

In Vitro DNA and RNA Syntheses with Purified Proteins

RNA synthesis on SS templates with RNA polymerase was conducted in a reaction mixture (25 μl) containing 40 mM HEPES–KOH (pH 7.6), 40 mM potassium glutamate, 10 mM magnesium acetate, 4 mM dithiothreitol, 80 μg/ml bovine serum albumin, 0.5 mM each of ATP, CTP, and GTP, 10 μM of UTP with 2 μCi of [α-^{32}P]UTP, 20 units of RNasein inhibitor (Promega), 270 ng of RNA polymerase holoenzyme, and 0.2 μg of SS template. When SSB was included, it was incubated with template DNA for 10 min on ice prior to addition of RNA polymerase. Run-off transcription was carried out in the same reaction mixture except that the template was 0.1 μg of isolated linear DNA fragment. For synthesis of ''cold'' RNA, radioactive ribonucleotide was omitted and all the nucleotides were present at 0.5 mM. For DNA synthesis, the above reaction mixture was supplemented with 20 μM each of 4 dNTPs with 5 μCi of [α-^{32}P]TTP and 70 ng of DNA polymerase III* supplemented with 75 ng of β subunit, and ribonucleotide cocktail was replaced by 2 mM ATP and 0.5 mM each of CTP, GTP, and UTP. Incubation was at 30°C for 10 min for both RNA and DNA syntheses. To measure the amount of RNA synthesis, 1 μl of the reaction mixture was spotted onto DE81 paper, followed by extensive washing in 0.5 M sodium phosphate buffer (pH 7.0). DNA synthesis was quantitated by measuring acid-insoluble radioactivity as above.

Analysis of In Vitro RNA and DNA Products

To analyze the RNA products on a denaturing polyacrylamide gel, the reaction mixtures were treated with phenol after addition of 200 μl of TE (10 mM Tris–Cl [pH 7.5], 1 mM EDTA), and RNA was precipitated with ethanol, washed, and dried. The pellet was resuspended in 5 μl of 80% formamide, warmed at 80°C for 3 min, and applied onto a gel.

To analyze the DNA products on gel electrophoresis, the reaction mixtures were phenol-treated after addition of 200 μl of the "stop solution" containing 10 mM EDTA and 0.3 M sodium acetate and nucleic acids were precipitated by ethanol, washed with 70% ethanol, and dried. For analyses on an alkaline agarose gel, the products were resuspended in an appropriate volume of 50 mM NaOH and 1 mM EDTA. Alkaline agarose gels were run in 30 mM NaOH and 1 mM EDTA. To analyze the products on a denaturing polyacrylamide gel, they were resuspended in 5 μl of formamide dye (80% formamide, 50 mM Tris–borate [pH 8.0], 1 mM EDTA, 0.1 % [w/v] xylene cyanol, 0.1% [w/v] bromophenol blue), heated at 95°C for 5 min, and quickly cooled on ice before being applied to a gel. Polyacrylamide gel electrophoresis was conducted in 0.5× TBE (Tris–borate–EDTA) buffer (Sambrook et al., 1989).

Mapping of 5′ Ends of Nascent RNA and DNA Chains by Restriction Digestion

Products of in vitro DNA replication were purified as above and were digested with a restriction enzyme and the nucleic acids were precipitated with ethanol after phenol treatment. The digested products were resuspended in 20 μl of 50 mM NaOH and 1 mM EDTA and were applied onto alkaline agarose gels. Alternatively, they were resuspended in 5 μl of formamide dye, heated at 95°C for 3 min, and applied onto a polyacrylamide gel containing 8 M urea. Size markers were generated by filling-in reaction of λ DNA digested with HindIII or end-labeling (phosphorylation of 5′ hydroxyl groups) of HaeIII-digested øX174 RF DNA in the presence of [α-^{32}P]dCTP or [γ-^{32}P]ATP, respectively.

Mapping of 5′ Ends of Nascent RNA by Primer Extension

RNA synthesized in vitro with purified proteins and "cold" ribonucleotides were purified by phenol extraction and ethanol precipitation. 5′-end-labeled reverse primer (1.5 pmol, 24-mer) in 10 μl was added to the precipitated RNA and hybridization was carried out by incubating the mixture at 70°C for 10 min and at 37°C for 5 min. Additional components were added so that the final reaction mixture (50 μl) contained 50 mM Tris–Cl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, and 0.5 mM each of four deoxynucleotides. After one hundred units of reverse transcriptase (SuperScript II, GIBCO–BRL) was added, the reaction mixtures were incubated at 42°C for 1 hr. The products were ethanol precipitated, were resuspended in formamide dye, and were analyzed on a polyacrylamide gel containing 8 M urea.

DNase I Protection Assays and P1 Nuclease Mapping

An oligonucleotide (17-mer ''−40'' universal primer, New England Biolabs, Inc.) was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled oligonucleotide (3 pmol) was mixed with 2 μg (0.8 pmol circle) of M13(F*rpo*) in an annealing solution (100 μl) containing 40 mM HEPES–KOH (pH 7.6), 5 mM EDTA, and 100 mM potassium glutamate, which was incubated at 80°C for 3 min, gradually cooled down to 37°C, and kept at this temperature for 2 hr. The SS–primer oligonucleotide hybrid was separated from the free oligonucleotides by spin-column gel filtration on Bio-Gel A15m (Bio-Rad; column volume, 1 ml). The reaction mixtures for DNase I or P1 nuclease digestion (25 μl) contained 40 mM HEPES–KOH (pH 7.6), 100 mM potassium glutamate, 10 mM magnesium acetate, 4 mM dithiothreitol, 80 μg/ml bovine serum albumin, 0.4 mM calcium chloride, and 40 fmol of the hybrid with or without protein(s) of indicated amount. After incubation at 30°C for 10 min, 0.8 ng of DNase I (Takara) was added and incubation was continued at 30°C for 1 min. For P1 nuclease mapping, P1 nuclease (Pharmacia Biochemicals, Inc.) of indicated amount was added to the SS–primer complex and was incubated at 30°C for 1 min. After the reaction was terminated by addition of 400 μl of the "stop solution," the nucleic acids were treated with phenol and were precipitated by adding 1 ml of ethanol and 1 μl of etachin-mate (Nippon-gene). The pellet was washed with 70% ethanol and dried. DNA chains were elongated from the primer terminus by incubation in 5 μl of the elongation mixture containing 40 mM HEPES–KOH (pH 7.6), 10 mM magnesium acetate, 40 mM potassium glutamate, 1 mM dithiothreitol, 0.1 mM each of four deoxynucleotides, and 1 unit of Sequenase (United States Biochemicals, Inc.) for 10 min at 37°C. After addition of 5 μl of formamide dye, reaction mixtures were incubated at 95°C for 3 min and were applied onto a polyacrylamide gel containing 8 M urea along with dideoxy sequencing reactions conducted on the same template with the same primer.

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